



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US94/12899 <b>(22) International Filing Date:</b> 9 November 1994 (09.11.94) <b>(30) Priority Data:</b> 08/150,368 9 November 1993 (09.11.93) US <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 08/150,368 (CIP) Filed on 9 November 1993 (09.11.93) <b>(71) Applicant (for all designated States except US):</b> THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Suite 300, 3700 Market Street, Philadelphia, PA 19104-3147 (US). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> LEE, Virginia [US/US]; 776 South 5th Street, Philadelphia, PA 19147 (US). <b>(74) Agents:</b> CALDWELL, John, W. et al.; Woodcock Washburn Kurtz Mackiewicz & Norris, One Liberty Place - 46th floor, Philadelphia, PA 19103 (US).		<b>(81) Designated States:</b> CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> COMPOSITIONS AND METHODS FOR PRODUCING AND USING HOMOGENEOUS NEURONAL CELL TRANSPLANTS  <b>(57) Abstract</b> <p>Methods of treating individuals suspected of suffering from diseases, conditions or disorders of the Central Nervous System which comprise implanting stable, homogeneous post-mitotic human neurons into the individual's brain are disclosed. Methods of treating individuals suspected of suffering from injuries, diseases, conditions or disorders characterized by nerve damage which comprise implanting stable, homogeneous post-mitotic human neurons at or near a site of said nerve damage. Pharmaceutical compositions comprising stable, homogeneous post-mitotic human neurons and a pharmaceutically acceptable medium are disclosed. Methods of generating non-human animal models of human CNS diseases, conditions or disorders which comprise implanting stable, homogeneous post-mitotic human neurons into the brain of a non-human animal are disclosed. Non-human animals comprising stable, homogeneous post-mitotic human neurons implanted in their brain are disclosed.</p>		

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**COMPOSITIONS AND METHODS FOR PRODUCING AND USING  
HOMOGENOUS NEURONAL CELL TRANSPLANTS**

**FIELD OF THE INVENTION**

The present invention relates to compositions useful  
5 for and methods of transplanting stable, homogeneous  
populations of neuron cells into non-human animals in order to  
generate non-human animal models useful to study human  
diseases, conditions and disorders. The present invention  
relates to compositions useful for and methods of transplanting  
10 stable, homogeneous populations of neuron cells into  
individuals in order to treat or prevent diseases, conditions  
and disorders, especially those characterized by loss, damage  
or dysfunction of the brain and/or loss, damage or dysfunction  
of an individuals neurons at other sites in the individual's  
15 body. This application is related to U.S. Patent Application  
Serial Number 08/150,368 filed November 9, 1993, U.S. Patent  
Application Serial Number 08/170,668 filed December 17, 1993,  
U.S. Patent Application Serial Number 07/911,980 filed July 10,  
1992, and U.S. Patent Application Serial Number 07/780,715,  
20 filed October 21, 1991, now U.S. Patent Number 5,175,103 issued  
December 29, 1992, which are each incorporated herein by  
reference. This invention was made in the course of research  
sponsored by the NIH grant number NS18616. The United States  
Government has certain rights in this invention.

**25 BACKGROUND OF THE INVENTION**

The transplantation of major categories of central  
nervous system (CNS) cells (i.e. neurons, astrocytes) or CNS  
tissue fragments offers opportunities to study the

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developmental biology and immunological properties of these cells, to create animal models of CNS diseases such as Alzheimer's disease and to develop alternative strategies for the treatment of relentlessly progressive neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and hereditary ataxia as well as to study other diseases, conditions and disorders characterized by loss, damage or dysfunction of neurons including transplantation of neuron cells into individuals to treat individuals suspected of suffering from such diseases, conditions and disorders. Indeed, recent pioneering efforts to utilize human fetal mesencephalic tissue grafts to ameliorate the extrapyramidal manifestations of drug induced and idiopathic Parkinson's disease emphasize the potential of transplanted human CNS tissues for the treatment of human neurodegenerative diseases (Freed, C.A., et al. 1992 *New Engl. J. Med.* 327:1549-1555; Spencer, D.D. et al. 1992 *New Engl. J. Med.* 327:1541-1548; and Widner, H., et al. 1992 *New Engl. J. Med.* 327:1556-1563). However, the results of these efforts have not been completely satisfactory.

The immortalization of CNS progenitor cells using constructs containing temperature sensitive promoters has enabled transplantation of genetically engineered precursors of neurons and glia, but brain grafts of these progenitors have given rise to mixed populations of glial and neuronal progeny (Cattaneo, E., and R. McKay 1991 *TINS* 14:338-340; Renfranz, P.J., et al. 1991 *Cell* 66:713-729; Snyder, E.Y., et al. 1992 *Cell* 68:33-51). An alternative strategy has been to use neuron-like transformed cell lines obtained from tumors of the CNS, but neoplastic neuron-like cells usually cannot be induced to permanently exit the cell cycle or they develop into tumors when transplanted into the rodent brain (Fung, K.-F. et al. 1992 *J. Histochem. Cytochem.* 40:1319-1328; Trojanowski, J.Q., et al. 1992 *Molec. Chem. Neuropathol.* 17:121-135; and Wiestler, O.D. et al. 1992 *Brain Pathol.* 2:47-59). A slowly dividing human neuronal cell line obtained from a child with unilateral

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megalencephaly was shown to exhibit a neuron-like phenotype in culture but grafts of these cells in the rodent CNS showed a mixture of neuronal and mesenchymal phenotypic properties (Poltorak, M., et al. 1992 *Cell Transplant* I:3-15).

5           There is a need for a method of generating animal models of CNS diseases and disorders by transplanting neurons into the brains of such animals to produce conditions which resemble or mimic CNS diseases, conditions or disorders.

          There is a need for animal models of CNS diseases and  
10 disorders by transplanting neurons into the brains of such animals to produce conditions which resemble or mimic CNS diseases, conditions or disorders.

          There is a need for a method of treating individuals suspected of suffering from CNS diseases, conditions or  
15 disorders by transplanting neurons in order to replace or introduce cells whose presence reverses or impedes the pathology associated with the disease being treated.

          There is a need for a method of treating individuals suspected of suffering from neuron damage caused by stroke or  
20 injury such as head trauma, nerve injury or spinal injury by transplanting neurons in order to replace cells damaged by stroke or an injury.

#### SUMMARY OF THE INVENTION

          The present invention relates to a method of treating  
25 an individual suspected of suffering from a disease, condition or disorder characterized by the damage or loss of neurons which comprises implanting a sample from a culture of at least 95% pure, stable, homogeneous post-mitotic human neurons into the individual at or near the site of the damage or loss.

30           The present invention relates to a method of treating an individual suspected of suffering from an injury, disease, condition or disorder to the Central Nervous System which comprises implanting a sample from a culture of at least 95% pure, stable, homogeneous post-mitotic human neurons into the  
35 individual's brain.

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The present invention relates to a method of treating an individual suspected of suffering from an injury, disease, condition or disorder to the spinal cord which comprises implanting a sample from a culture of at least 95% pure, stable, homogeneous post-mitotic human neurons into the individual's spinal column.

The present invention relates to a method of treating an individual suspected of suffering from an injury, disease, condition or disorder to nerve cells which comprises implanting a sample from a culture of at least 95% pure, stable, homogeneous post-mitotic human neurons into the individual's body at the site of nerve dysfunction or damage.

The present invention relates to a pharmaceutical composition that comprises a sample from a culture of at least 95% pure, stable, homogeneous post-mitotic human neurons and a pharmaceutically acceptable medium.

The present invention relates to a method of generating a non-human animal model for a human disease, condition or disorder of the Central Nervous System comprising implanting a sample from a culture of at least 95% pure, stable, homogeneous post-mitotic human neurons into a non-human animal.

The present invention relates to a non-human animal comprising a sample from a culture of at least 95% pure, stable, homogeneous post-mitotic human neurons implanted in its brain, nervous system or spinal column.

#### DESCRIPTION OF DRAWINGS

Figure 1A, Figure 1B, Figure 1C, Figure 1D, Figure 1E, Figure 1F, Figure 1G and Figure 1H contain photomicrographs of NT2N graft in the hippocampus (dentate gyrus and polymorph layer) 4 weeks post-transplant probed with various monoclonal antibodies.

Figure 2A, Figure 2B and Figure 2D show photomicrographs of three different NT2N grafts in the subcortical white matter and the dorsal diencephalon (Figure 2C) 2-4 weeks post-transplant stained with Cresyl Violet

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(Figure 2A, Figure 2C and Figure 2D) or the MAb (ED1) to macrophages (Figure 2B).

Figure 3A, Figure 3B, Figure 3C, Figure 3D, Figure 3E, Figure 3F, Figure 3G and Figure 3H contain photomicrographs of an NT2N graft in the subcortical white matter at 4 weeks post-transplant probed with MAbs and counterstained with hematoxylin.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to compositions and methods relating to transplanting neurons into either individuals who are suspected of suffering from an injury, disease, disorder or condition or into non-human animals to generate a non-human animal model of a human disease, disorder or condition. The neurons used in the methods of the present invention are at least 95% pure, stable, homogeneous post-mitotic human neurons. Optionally, the neurons may be comprise exogenous genetic material. The neurons used in the methods of the present invention are genotypically and phenotypically homogenous.

As used herein, the term "sample" is meant to refer to one or more cells. In preferred embodiments, a sample contains a plurality of cells. According to the present invention, a sample from a culture of at least 95% pure, stable, homogeneous post-mitotic human neurons is implanted into either a non-human animal or a human. Accordingly, the methods of the present invention relate to the implantation of one or more cells from a culture of at least 95% pure, stable, homogeneous post-mitotic human neurons into either a non-human animal or a human.

As used herein, the term "at or near a site of said nerve damage" is meant to refer to the location where nerve cells are implanted in order to replace destroyed, damaged or dysfunctional nerve cells and/or restore function resulting from destroyed, damaged or dysfunctional nerve cells. The location is defined as being a site where such implanted cells can develop as replacement cells for destroyed, damaged or dysfunctional nerve cells and make the necessary linkages to

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restore function lost due to destroyed, damaged or dysfunctional nerve cells.

As used herein, the term "exogenous genetic material" refers to genomic DNA, cDNA, synthetic DNA and RNA, mRNA and antisense DNA and RNA which is introduced into the cell or an ancestor cell. The exogenous genetic material may be heterologous or an additional copy or copies of genetic material normally found in the individual or animal. When cells are used as a component of a pharmaceutical composition in a method for treating human injuries, diseases, conditions or disorders, the exogenous genetic material that is used to transform the cells may encode proteins selected as therapeutics used to treat the individual and/or to make the cells more amenable to transplantation. When cells are used in a method for generating non-human animal models of human CNS diseases or disorders, the exogenous genetic material that may be incorporated into the cells may encode proteins selected to create conditions in the non-human animal which simulates or resembles conditions in a human suffering from CNS disease, condition or disorder to be modeled.

The exogenous genetic material is preferably provided in an expression vector which includes the coding region of a protein whose production by the cells is desirous operably linked to essential regulatory sequences such that when the vector is transfected into the cell, the exogenous genetic material is capable of being expressed within the cell.

According to some embodiments of the present invention, a sample from a culture of pure, stable, homogeneous post-mitotic human neurons is transplanted into an individual being treated for a CNS injury, disease, condition or disorder. These cells essentially replace and/or function in place of endogenous damaged, dead, non-functioning or dysfunctioning cells. Thus, in the case of an individual suffering from an injury, disease, condition or disorder characterized by loss, damage or dysfunction of neurons such as, for example, diseases associated with nerve damage or spinal injury, the cells are transplanted into a site in the individual where the



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transplanted cells can function in place of the lost, damaged or dysfunctional cells and/or produce products needed to improve or restore normal functions that have been reduced or lost due to the lack of such products endogenously produced in the individual. In the case of an individual suffering from a CNS injury, disease, condition or disorder characterized by loss, damage or dysfunction of neurons in the brain, the cells are transplanted into the brain of the individual. The transplanted cells function in place of the lost, damaged or dysfunctional cells and/or produce products needed to improve or restore normal brain functions that have been reduced or lost due to the lack of such products endogenously produced in the individual.

According to some embodiments of the present invention, a sample from a culture of pure, stable, homogeneous post-mitotic human neurons are transplanted into the individual being treated for a disease, condition or disorder in order to provide living neurons which produce desired substances. The transplanted cells may produce specific products that, when present at or near the site of implantation in the treated individual, reverses or impedes the pathology associated with the disease, condition or disorder being treated.

According to some embodiments of the present invention, a sample from a culture of pure, stable, homogeneous post-mitotic human neurons are transplanted into a non-human animal in order to provide a model for a human CNS disease, condition or disorder. The transplanted cells may produce products that result in the development of conditions which are similar to or mimic the pathology of a CNS disease or condition.

The method may be used to treat individuals suffering from injuries, diseases, conditions or disorders characterized by the loss, damage or dysfunction of endogenous cells. The method may be used to treat individuals suffering from stroke, spinal injury or other injuries, conditions or disorders associated with neuron damage or death. CNS diseases and disorders which may be treated by practicing the methods of the

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present invention include any disease of the CNS which is characterized by the loss, damage or dysfunction of endogenous cells, the symptoms of which may be reversed or reduced in severity by providing neurons that can replace such cells and  
5 produce products needed for proper function or needed to counteract the presence of compounds that are not normally present or present at abnormal levels. The present invention is useful for the treatment of progressive neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease,  
10 Huntington's disease, amyotrophic lateral sclerosis and hereditary ataxia as well as neurological conditions such as strokes and nerve injuries. The present invention is useful to treat diseases by serving as a delivery system to produce and disseminate active proteins and other active compounds needed  
15 for proper brain function.

A pharmaceutical composition according to the present invention useful for treating individuals suffering from injuries, diseases, conditions or disorders characterized by the loss, damage or dysfunction of endogenous cells comprises  
20 a sample from a culture of pure, stable, homogeneous post-mitotic human neurons and a pharmaceutically acceptable medium. The neurons used in the present invention must be a stable, homogeneous culture of post-mitotic human neurons that is at least 95% pure. The neurons used in the present invention may  
25 be transfected with exogenous genetic material.

The exogenous genetic material used to transform the cells may encode proteins selected as therapeutics for delivery to the brain of the treated individual. Protein products encoded by transfected genetic material include, but are not  
30 limited to, those leading to production of neurotransmitters (e.g. tyrosine hydroxylase) as well as neurotrophic substances such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDGF), basic fibroblast growth factor (bFGF) and glial-derived growth factor (GDGF). In addition, tumor suppressor  
35 genes such as P53 and thrombospondin can be incorporated into the cells.

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According to another embodiment of the present invention, a sample from a culture of pure, stable, homogeneous post-mitotic human neurons are transplanted into the brain of a non-human animal in order to generate a non-human animal  
5 model of a human CNS disease, condition or disorder. The presence of the cells bring about changes in the animal's brain such that animal develops features which resemble or mimic the characteristics of the human CNS diseases, conditions or disorders. The transplanted cells produce specific products  
10 that, when present in the brain of the animal, give rise to conditions which resemble or mimic the pathology associated with the disease being modeled. The cells used to generate the non-human animal models according to the present invention useful for treating CNS diseases comprises a sample from a  
15 culture of pure, stable, homogeneous post-mitotic human neurons and a pharmaceutically acceptable medium. The neurons used in the present invention must be a stable, homogeneous culture of post-mitotic human neurons that is at least 95% pure.

CNS diseases and disorders which may be modeled by  
20 practicing the methods of the present invention include any disease of the CNS which is characterized by endogenous dead, non-functioning or dysfunctioning cells, particularly those characterized by cells producing proteins not normally associated with the cells or producing normal proteins at  
25 abnormal levels. Thus, the transplantation into the brain of an animal of cell which produce proteins associated with a human CNS disease gives rise to conditions which resemble or mimic the characteristics associated with the pathology of the disease or disorder being modeled. The present invention is  
30 useful to generate non-human animal models of progressive neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, hereditary ataxia, and motor neuron and Lewy body disease. Many different genes are implicated in these diseases  
35 such as normal and mutated amyloid precursor genes, genes encoding kinases, phosphatases, normal and mutated superoxide dismutase, neurofilament proteins and apolipoprotein 4. In

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addition, specific oncogenes responsible for certain types of cancer can be incorporated to generate animal models for such cancer using the NT2 derived cells.

In some embodiments of the present invention, the  
5 neurons used may be produced by a method described in U.S. Patent Number 5,175,103 issued December 29, 1992, which teaches a method for obtaining >95% pure postmitotic human neurons (termed NT2N cells) from a human teratocarcinoma cell line (termed NTera2/clone PI or NT2 cells); following treatment of  
10 the NT2 cells with retinoic acid (RA). In addition to providing a model system for a wide range of biochemical, molecular biological and morphological studies of neurons *in vitro*, the stable, homogeneous population of pure human neurons may be used in *in vivo* transplants in order to generate animal  
15 models of CNS diseases and disorders or they may be used in *in vivo* transplants into the brains of individuals suffering from CNS diseases or disorders as therapeutics/prophylactics to introduce neurons which are capable of producing products that reverse or impede the pathology associated with CNS diseases or  
20 disorders afflicting the individual.

The NT2 cell line is unique among all other teratocarcinoma cell lines that are capable of differentiating into neurons, glia and mesenchymal cells, because the NT2 cells appear to correspond to progenitor cells, the progeny of which  
25 are restricted to the neuronal lineage (Abramham, I. et al. 1991 *J. Neurosci. Res.* 28:29-39; Andrews, P.W., et al. 1981 *Tissue Antigens* 17:493-500; Andrews, P.W. et al 1984. 1984 *Lab. Invest.* 50 147-162; Andrews, P.W. 1987. *Devel. Biol.* 103:285-293; Kleppner, S.R., et al 1992 *Soc. Neurosci. Abst.*  
30 18:782; Lee, V.M.-Y. and P.W. Andrews 1986 *J. Neurosci.* 6:514-521; and, Younkin, D.P. et al. 1993 *Proc. Natl. Acad. Sci. U.S.A.* 90:2174-2178). Further characterization of the NT2N cells has shown that these cells most closely resemble CNS neurons (Pleasure, S.J., and V.M. -Y. Lee. 1993 *J. Neurosci.*  
35 *Res.* In press; and Pleasure, S.J., et al. 1992. *J. Neurosci.* 12:1802-1815). The NT2N cells exhibit other interesting properties of CNS neurons, i.e. they express almost exclusively

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the 695 amino acid long amyloid precursor protein (APP), produce and secrete the  $\beta$ -amyloid or A4 ( $\beta$ /A4) peptide found in Alzheimer's disease amyloid plaques and bear glutamate receptor channels on their cell surface.

5           The neurons used in the present invention may be transfected with exogenous genetic material. If produced as described in U.S. Patent Number 5,175,103, the neurons used in the present invention may be transfected with genetic material prior to induction of differentiation. Methods of transfection  
10 are well known and taught in the above-referenced patent. The exogenous genetic material used to transform the cells may encode proteins whose presence within cell of the brain are associated with human diseases, disorders or conditions. Protein products encoded by transfected genetic material  
15 include, but are not limited to, normal and mutated amyloid precursor, kinases, phosphatases, normal and mutated superoxide dismutase, neurofilament proteins and apolipoprotein 4 as well as neurotransmitters (e.g. tyrosine hydroxylase) and neurotrophic substances such as nerve growth factor (NGF),  
20 brain-derived neurotrophic factor (BDGF), basic fibroblast growth factor (bFGF) and glial-derived growth factor (GDGF).

          The exogenous genetic material used to transfect the cells is preferably provided in a vector which includes essential regulatory sequences operably linked to coding  
25 sequences such that the transfected genetic material is capable of being expressed within the cell.

          Expression vectors that encode exogenous genetic material comprise a nucleotide sequence that encodes a protein to be produced operably linked to regulatory elements needed  
30 for gene expression. Accordingly, incorporation of the DNA or RNA molecule into the neuron cell results in the expression of the DNA or RNA encoding the protein and thus, production of the protein.

          The exogenous genetic material that includes the  
35 nucleotide sequence encoding the protein operably linked to the regulatory elements may remain present in the cell as a functioning episomal molecule or it may integrate into the

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cell's chromosomal DNA. Exogenous genetic material may be introduced into cells where it remains as separate genetic material in the form of a plasmid. Alternatively, linear DNA which can integrate into the chromosome may be introduced into  
5 the cell. When introducing DNA into the cell, reagents which promote DNA integration into chromosomes may be added. DNA sequences which are useful to promote integration may also be included in the DNA molecule. Alternatively, RNA may be introduced into the cell.

10           The necessary elements of an expression vector include a nucleotide sequence that encodes a protein and the regulatory elements necessary for expression of that sequence in the cells. The regulatory elements are operably linked to the nucleotide sequence that encodes the protein to enable  
15 expression. The nucleotide sequence that encodes the protein may be cDNA, genomic DNA, synthesized DNA or a hybrid thereof or an RNA molecule such as mRNA.

          The regulatory elements necessary for gene expression include: a promoter, an initiation codon, a stop codon, and a  
20 polyadenylation signal. It is necessary that these elements be operable in the neurons. Moreover, it is necessary that these elements be operably linked to the nucleotide sequence that encodes the protein such that the nucleotide sequence can be expressed in the neuron cells and thus the protein can be  
25 produced.

          Initiation codons and stop codon are generally considered to be part of a nucleotide sequence that encodes the protein. However, it is necessary that these elements are functional in the neurons.

30           Similarly, promoters and polyadenylation signals used must be functional within the neuron cells.

          Examples of promoters useful to practice the present invention include but are not limited to cytomegalovirus promoter, particular the immediate early promoter, SV40  
35 promoter and retroviral promoters.

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An examples of polyadenylation signals useful to practice the present invention includes but is not limited to SV40 polyadenylation signal.

An additional element may be added which serves as a target for cell destruction if it is desirable to eliminate transplanted cells for any reason. An expressible form of a herpes thymidine kinase (tk) gene can be included in the exogenous genetic material. When the exogenous genetic material is introduced into the neuron, tk will be produced. If it is desirable or necessary to kill the transplanted cells, the drug gangcyclovir can be administered to the individual and that drug will cause the selective killing of any cell producing tk. Thus, a system can be provided which allows for the selective destruction of transplanted cells.

In order for exogenous genetic material in an expression vector to be expressed, the regulatory elements must be operably linked to the nucleotide sequence that encodes the protein. Accordingly, it is necessary for the promoter and polyadenylation signal to be in frame with the coding sequence. In order to maximize protein production, regulatory sequences may be selected which are well suited for gene expression in the neuronal cells. Moreover, codons may be selected which are most efficiently transcribed in the cell. One having ordinary skill in the art can produce exogenous genetic material as expression vectors which are functional in neurons.

Neurons may be transplanted into individuals suspected of suffering from injuries, diseases, conditions or disorders characterized by the damage or loss of neurons at the site of such neuron injury or loss by direct grafting of neurons at the site of neuron injury or loss. Neurons may be transplanted into the brains of individuals suspected of suffering from CNS diseases, conditions or disorders by direct grafting of neurons into the brains of such individuals. Additionally, neurons may be transplanted into the brains of individuals suffering form head trauma or strokes. Individuals suspected of or identified as suffering from diseases, conditions, disorders, or injuries rendering neurons in the brain damaged, destroyed or

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dysfunctional may be treated by implantation of neurons to replace or compensate for the loss of neuron function due to the destruction or dysfunction of endogenous neurons.

In some embodiments,  $1 \times 10^3$  to  $1 \times 10^6$  neurons are  
5 implanted. In some embodiments,  $5-10 \times 10^4$  neurons are  
implanted. Two techniques have been used for neural  
transplantation, the first comprises stereotaxic surgery in  
which a neuron cell suspension is implanted into the brain, the  
second in which the cells are grafted into the brain by  
10 microsurgery. Techniques for transplanting neural tissue are  
disclosed in: Backlund, E.-O. et al., (1985) *J. Neurosurg.*  
**62**:169-173; Lindvall, O. et al. (1987) *Ann. Neurol.* **22**:457-468;  
and Madrazo, I. et al. (1987) *New Engl. J. Med.* **316**:831-834;  
each of which is incorporated herein by reference.

15 Neurons may be implanted into the spinal cord at or  
near the site of nerve damage from disease or injury. The  
implanted cells further differentiate into motor neurons,  
thereby replacing or reconnecting nerves at the site of damage.  
In some embodiments, the injury is to a motor neuron which is  
20 part of the spinal cord. In some embodiments, the injury is to  
a motor neuron outside the spinal column. Neurons of the  
invention are implanted at the site of the nerve cell injury,  
i.e. in proximity to the injured cell or cells at a location  
where differentiation of implanted cells can replace nerve  
25 function and reconnect nerves of the individual to remedy or  
otherwise ameliorate the injury. The neurons are implanted in  
a location that allows processes which develop therefrom to  
substitute for the processes of the damaged nerve, thereby  
repairing the damaged nerve network.

30 Neurons may be transplanted into the brains of non-  
human animals by injection of neurons into one hemisphere using  
a stereotaxic instrument and a hand-held 10 ml Hamilton  
syringe. Aliquots of  $1 \times 10^3$  to  $1 \times 10^6$  neurons are injected  
into the adult and neonatal rats. In some embodiments,  $5-10 \times$   
35  $10^4$  neurons are injected. For the adult rats, cells are  
injected stereotaxically into cerebral cortex, subjacent white



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matter or hippocampus at one site in a single hemisphere of each rat.

The present invention is further illustrated by the following examples, which are not intended to be limiting in any way.

#### EXAMPLES

##### Example 1

Studies of NT2N cells transplanted into the brains of nude mice show that these NT2N cells integrate as neurons into the brains of immunodeficient nude mice where they survive >12 months without evidence of rejection or tumor formation. Furthermore, the transplantation of NT2N neurons in cyclosporine-treated and untreated immunocompetent Sprague-Dawley rats has been performed and survival of the cells has been observed.

The following is a review of experiments demonstrating implantation of transfected neuronal cells into immunocompetent animals.

#### Materials and Methods

Culture of NT2 cells and generation of NT2N neurons were performed essentially as described in U.S. Patent Number 5,175,103. Briefly, NT2 cells were cultured using standard techniques and were passaged 1:3 twice per week in OptiMEM with 5% fetal bovine serum and penicillin/streptomycin. NT2 cells were induced to differentiate into neurons by administration of 10  $\mu$ m retinoic acid (RA), which was replenished twice weekly, for 5 weeks at which time the cells were replated to establish Replate 1 cells. Highly differentiated NT2N cells were then obtained following two subsequent replate manipulations (designated Replate 2 and Replate 3) at which time the NT2N cells were >99% pure. Freshly harvested aliquots of Replate 3 NT2N neurons were washed three times in buffer and then used in the transplantation studies described here.

Additionally, in experiments conducted in 2 rats, previously frozen aliquots of NT2N cells were thawed immediately prior to injection into the CNS.

Implantation of NT2N Cells Into Rat Brain:

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Adult (170-280 gm) female Sprague/Dawley rats were anesthetized by intraperitoneal injections of Ketamine (87 mg/kg) and Xylazine (13 mg/kg), prepared for surgery and placed in a stereotaxic instrument (Kopf, Tujunga, CA). Neonatal  
5 (postnatal day 5) female Sprague/Dawley rats were anesthetized by hypothermia during the injection of NT2N cells into one hemisphere using a stereotaxic instrument and a hand-held 10  $\mu$ l Hamilton syringe. Aliquots of 5-10  $\times 10^4$  NT2N cells were injected into the adult and neonatal rats. For the adult rats,  
10 NT2N cells were injected stereotaxically into cerebral cortex, subjacent white matter or hippocampus at one site in a single hemisphere of each rat. A total of 68 rats were used in this study (see Table 1).

The stereotaxic injection sites were determined using  
15 system B of Pellegrino et al. (Pellegrino, L.J., et al. 1979. A Stereotaxic Atlas Of The Rat Brain, Plenum Press, New York) and all of the injections were performed by injecting 2  $\mu$ l of a suspension of the NT2N cells over 5 min. After the injection, the needle was left in place for another 5 min. and  
20 then slowly removed. The viability of the NT2N cells before they were injected was monitored microscopically using Trypan blue exclusion. Similar procedures were used to monitor the viability of residual, uninjected NT2N cells after the transplantation procedure had been completed.

25 A subset of the adult rats (N=13) implanted with NT2N cells were treated daily by the oral (N=8; using a gavage tube) or subcutaneous (N=5) administration of cyclosporine (7-10 mg/kg per day) for the duration of their survival post-transplantation.

30 Following different post-transplantation survival times, the rats were deeply anesthetized and sacrificed by perfusion with phosphate buffered saline (to wash out red blood cells and serum proteins) followed by 70% ethanol and 150mM NaCl. The brains were removed and fixed by overnight immersion  
35 in 70% ethanol and 150mM NaCl. The post-transplant survival times ranged from 4 days to 21 weeks as summarized in Table 1.

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Table 1 summarizes data on the number of adult (with and without subcutaneous or oral cyclosporine treatment) and neonatal rats used for transplantation as well as the survival times post-transplantation for each group of rats (left and middle columns). The number of rats with viable NT2N grafts is shown in the far right column. The number of rats treated with subcutaneous (sc) cyclosporine is indicated in parentheses.

#### Immunohistochemical Procedures:

The methods for tissue processing and light microscopic immunohisto-chemical analysis are well known. Antibodies were used for the immunohistochemical characterization of the NT2N grafts. Both monoclonal and polyclonal antibodies to neuronal and glial cytoskeletal proteins and other polypeptides that have been shown to serve as molecular signatures of the neuronal or glial phenotype were selected to identify and characterize the NT2N grafts. These antibodies have been extensively characterized and their properties are summarized in Table 2.

Specifically, Table 2 summarizes the properties of the 27 different antibodies used in this study and their reactivity with NT2N cells grafted into the rat brain. The far left column indicates the polypeptide recognized by the antibody which is named in the second column. The third column gives the dilution or immunoglobulin concentration of each antibody as it was applied here. The fourth column indicates whether or not the antibody stained grafted NT2N cells (+ positive; - - negative; +/- - weak or equivocal staining). The antibodies are grouped together according to the cell types in which they are predominantly or exclusively expressed.

The abbreviations used in the first column of the Table 2 (in alphabetical order) are:

GFAP = Glial fibrillary acid protein;  
MAP2 = Microtubule-associated protein 2;  
MAP5 = Microtubule-associated protein 5;  
MBP = myelin basic protein;  
N-CAM = Neural-cell adhesion molecule;  
NF = Neurofilament;

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NF-L = Low molecular weight NF protein;

NF-M = Middle molecular weight NF protein;

NF-H = High molecular weight NF protein;

5 p75 NGFR = Low affinity (75 kD) nerve growth factor  
receptor;

P<sup>ind</sup> = Phosphate independent epitope in NF-L or NF-H;

P<sup>-</sup> = Non- or poorly phosphorylated epitope in NF-H or  
NF-M;

P<sup>+</sup> = Moderately phosphorylated epitope in NF-H;

10 P<sup>+++</sup> = Heavily phosphorylated epitope in NF-H;

PHF = Paired helical filaments in Alzheimer's disease  
neurofibrillary tangles.

Note that two antibodies (i.e. T3P and PHF1) to tau  
proteins recognize fetal tau and the abnormally phosphorylated  
15 tau proteins (at serine number 396 according to the numbering  
system for the 441 amino acid long tau protein) in PHFs (also  
known as A68 proteins), but not normal adult tau. Note that  
although the anti-CFAP and anti-macrophage MABs stained  
occasional reactive astrocytes and macrophages, respectively,  
20 that had infiltrated the graft, the NT2N cells themselves were  
not stained by these MABs.

## RESULTS

Specific Identification Of NT2N Grafts With Monoclonal  
Antibodies:

25 Figure 1A and Figure 1B contains photomicrographs of  
NT2N graft in the hippocampus (dentate gyrus and polymorph  
layer) 4 weeks post-transplant. Figure 1A shows a low power  
view of a Cresyl Violet stained section of the NT2N graft  
(delineated by the arrows). Figure 1B shows a low power view  
30 of the same NT2N graft stained with the human specific  
anti-N-CAM MAb (MOC 1). The asterisk lies above the portion of  
the graft containing the perikarya and simple dendritic arbor  
of the NT2N neurons while the arrow heads identify the axons  
emanating from the graft and extending in the mossy fiber  
35 pathway dorsal to pyramidal neurons in CA3. The region  
identified by the asterisk is shown at higher power in Figure  
1C and the segment of graft-derived axons located below the

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middle arrow head is shown at higher power in Figure 1D. Note that only the NT2N neurons and their processes are stained by this MAb. Figure 1A and Figure 1B are at the same magnification and the bar in Figure 1A = 100  $\mu$ m. Figure 1C and  
5 Figure 1D show a higher power views of the NT2N graft stained by the human specific anti-N-CAM MAb (MOCl). Note that the NT2N neurons and some of their dendrites (Figure 1C) as well as their axons (Figure 1D) are stained, but not the endogenous rodent N-CAMs. The photomicrographs in Figure 1C, Figure 1F,  
10 Figure 1G and Figure 1H are all at the same magnification and the bar in Figure 1C = 100  $\mu$ m, while Figure 1D and Figure 1E are taken at a slightly higher magnification and the bar in Figure 1C corresponds to 25  $\mu$ m in Figure 1D and Figure 1E. Figure 1E and Figure 1F show regions similar to those  
15 illustrated in Figure 1C and Figure 1D, respectively, in an adjacent section stained with the MAb RHdO20 (Figure 1D) to poorly phosphorylated NF-H/M and the MAb HO14 (Figure 1F) to moderately phosphorylated isoforms of NF-H. Figure 1G shows results obtained with a MAb to highly phosphorylated NF-H  
20 (RMO24) which stains only endogenous rat axons, but not the NT2N graft (arrows) despite the fact that RMO24 also recognizes human NF-H. The section shown in Figure 1H is adjacent to that seen in Figure 1F and it was probed with the MAb to GFAP. Some reactive astrocytes infiltrate the graft similar to the  
25 colonization of dorsal root ganglion grafts transplanted into rat brain, but most GFAP positive reactive astrocytes surround the graft. The sections in Figure 1B - Figure 1H were lightly counterstained with hematoxylin.

Figure 2A, Figure 2B, Figure 2C and Figure 2D show  
30 photomicrographs of three different NT2N grafts in the subcortical white matter (Figure 2A, Figure 2B and Figure 2D) and the dorsal diencephalon (Figure 2C) 2-4 weeks post-transplant stained with Cresyl Violet (Figure 2A, Figure 2C and Figure 2D) or the MAb (ED1) to macrophages (Figure 2B).  
35 Figure 2A and Figure 2B are adjacent sections of the same graft and the arrow heads identify the interface between the graft (above) and the subjacent white matter (below). Figure 2A and

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Figure 2B are at the same magnification and the bar in Figure 2A = 50  $\mu$ m. The arrows in Figure 2B identify ED1 positive macrophages in an area of the graft containing some NT2N neurons undergoing focal karyorrhexis. More extensive inflammation is seen around blood vessels in Figure 2C (arrow) at the margin of the graft (star) while more severe karyorrhexis of grafted NT2N cells is seen in another subcortical white matter NT2N graft shown in Figure 2D where the arrows identify accumulations of nuclear debris. Figure 2C and Figure 2D are at different magnifications and the bar in Figure 2A corresponds to 100  $\mu$ m in Figure 2C and to 30  $\mu$ m in Figure 2B.

Figure 3A, Figure 3B, Figure 3C, Figure 3D, Figure 3E, Figure 3F, Figure 3G and Figure 3H contain photomicrographs of an NT2N graft in the subcortical white matter at 4 weeks post-transplant probed with MABs and counterstained with hematoxylin. The section shown in Figure 3A was stained with the human specific anti-NF-H MAB (H014) which demonstrates the grafted perikarya and their dendrites in the NT2N transplant to the right in the figure. Labeled axons extend medially from the graft site to the left in this panel. Note that these axons cross the midline (star) within the corpus callosum. The section in Figure 3B, which was a section adjacent to that shown in Figure 3A, was probed with a MAB to human N-CAM (MOC1) which stains the somatodendritic domain of NT2N cells in the graft to the right in this figure as well as axons that cross the midline (star) to the left within the corpus callosum. Figure 3A and Figure 3B are at the same magnification and the bar in Figure 3A = 100  $\mu$ m. The axons in the corpus callosum seen in Figure 3A and Figure 3B are shown at higher power in Figure 3C and Figure 3D, respectively. These axons (arrows in Figure 3C and Figure 3D) cross the midline (star in Figure 3C and Figure 3D) to the hemisphere contralateral to the NT2N graft. Figure 3C and Figure 3D are at different magnifications and the bar in Figure 3C = 100  $\mu$ m while the same bar corresponds to 50  $\mu$ m in Figure 3D. In Figure 3E, the MAB to MAP2 (AP14) labels the somatodendritic domain of the grafted

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NT2N neurons. The cell body mass of the graft is identified by an asterisk and the overlying white matter (WM) is unstained. The somatodendritic domain of endogenous host neurons in the overlying cortex also are labeled in this section and labeled  
5 apical dendrites are most prominent at this magnification. Figure 3E is at the same magnification as Figure 3A and Figure 3B. In Figure 3F, the MAb to highly phosphorylated NF-H (RH024) does not stain the NT2N neurons and their processes in the graft (asterisk). However, endogenous axons in the  
10 surrounding white matter (WM) are labeled by this MAb thereby delineating the extent of the cell body mass and dendrites of this graft. The bar in Figure 3F = 100 $\mu$ m. The two photomicrographs shown in Figure 3G and Figure 3H are high power views of the NT2N grafts in adjacent sections stained  
15 with the anti-NF-L antiserum (Figure 3G) and the MAb (TA51) to moderately phosphorylated isoforms of NF-H (Figure 3H). Note that many of the NT2N neurons contain immunoreactive NF-L and NF-H (arrows in Figure 3G and Figure 3H, respectively) in their perikarya and processes. Additionally, endogenous rodent axons  
20 in the white matter (upper left in Figure 3G and Figure 3H) also are labeled by these antibodies. Figure 3G and Figure 3H are at the same magnification and the bar in Figure 3F corresponds to 50  $\mu$ m in Figure 3G and Figure 3H.

Although the grafts could be recognized in Cresyl  
25 Violet stained sections (Figure 1A, Figure 2A, Figure 2B and Figure 2D), the identification of transplanted NT2N cells in the rodent CNS was greatly facilitated by exploiting the restriction of certain polypeptides or epitopes contained within some of these polypeptides to human versus rat and  
30 mature versus immature CNS neurons. For example, MOC1, the monoclonal antibody (MAb) to human neural cell adhesion molecules (N-CAMs), was shown to recognize N-CAMs in the human NT2N neurons, but not the N-CAMs in the rat CNS (Figure 1B - Figure 1D). Indeed, the cytology of the NT2N cells was not  
35 sufficiently distinctive to allow recognition of the NT2N cells without the use of immunohistochemistry. Furthermore, axons arising from the NT2N grafts were only identifiable as graft

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derived when they were labeled with the human polypeptide specific antibodies described here (Figure 1B, Figure 1F, Figures 3A-3D). In addition to the anti-N-CAM MAb, the grafted NT2N cells also could be specifically identified with the MAb H014, an antibody that recognizes moderately phosphorylated isoforms of the middle (NF-M) molecular weight (Mr) neurofilament (NF) subunit in the human CNS and in NT2N cells, but not in the rodent CNS (Figure 1F). In contrast, RMO24 (Figure 1G) and RMO217, both of which are MAbs to the most heavily phosphorylated isoforms of the high (NF-H) Mr NF subunit that appear only in mature CNS neurons, immunostained NF-H in rodent CNS neurons, but these MAbs did not stain the human NT2N cells in the grafts described here. The inability of RMO24 and RMO217 to stain the grafted NT2N cells probably reflects the incomplete phosphorylation of NF-H in the grafted NT2N cells (which reflects the incomplete maturation of these grafted neurons), since both MAbs recognize phosphorylated NF-H extracted from the fully mature, human CNS. If the NT2N cells are allowed to survive for an extended period of time (i.e. >6 months) in the immunodeficient nude mouse brain, then the grafted NT2N neurons acquire the most heavily phosphorylated isoforms of NF-H and these fully mature grafted neurons are labeled by RMO24 and RMO217. However, grafted NT2N cells were only studied here for post-transplant survival times of <4 months, and both RMO24 and RMO217 strongly stained rat CNS neurons, but not the grafted NT2N cells, and MOC1 and H014 stained the NT2N grafts specifically and intensely, but not rat CNS neurons or other rat CNS cells. Thus, all 4 of these MAbs were used to screen sections from all 68 rats that received implants of the NT2N cells in order to specifically identify the surviving NT2N grafts. Additionally, a MAb (2.2B10) to glial fibrillary acidic protein (GFAP) stained reactive astrocytes surrounding the graft (Figure 1H) which also helped to delimit the NT2N grafts. Some of these reactive astrocytes infiltrated the NT2N grafts (Figure 1H) similar to the colonization of dorsal root ganglion grafts by reactive astrocytes transplanted into the rat brain. Screening the



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graft sites with this panel of MAbs provided a highly effective strategy for identifying grafted NT2N cells even when they existed as small clumps trapped in the leptomeninges or in the needle track dorsal to the injection site.

#### 5 Survival Of Grafted NT2N Cells:

Nearly all of the transplanted NT2N cells were accurately implanted into neocortex, subjacent white matter and hippocampus although a few also were detected in the diencephalon, the lateral ventricle or within the leptomeninges  
10 overlying the neocortical injection site. The number and disposition of the grafted NT2N cells varied from rat to rat, but NT2N grafts were identified immunohistochemically in 100% of adult (N = 5) and neonatal (N = 5) rats that survived for up to 2 weeks without cyclosporine treatment (see Table 1 for a  
15 summary of these and the following data on NT2N graft survival). This group of rats with viable NT2N grafts included 2 rats treated with cyclosporine that had been implanted with aliquots of previously frozen NT2N cells. At the next post-transplant survival interval, i.e. 4 weeks, 10/24 adult  
20 and 2/2 neonatal rats that were not treated with cyclosporine contained NT2N brain grafts (Table 1), and many of the transplanted cells resembled small stellate neurons morphologically and histochemically in Nissl stained preparations of the grafts. However, at subsequent  
25 post-transplant survival times, only 1 of 19 adult or neonatal rats that were not treated with cyclosporine contained identifiable, surviving NT2N neurons. These findings reflect rejection of the NT2N grafts rather than the cessation of expression of N-CAMs and NF proteins by the grafted NT2N cells.  
30 This conclusion is based upon 4 reasons:

1) inflammatory cells were detected in some of the viable grafts in association with cellular debris as early as 2 weeks post-transplantation (Figure 2C) and many of these inflammatory cells were identified as macrophages using the  
35 macrophage specific ED1 MAb (Figure 2B);

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2) cyclosporine prolonged the survival of all NT2N grafts in rats that received this agent by a subcutaneous route;

3) the maximum number of macrophages and inflammatory cells were noted to infiltrate the graft site at 2-4 weeks post-transplantation; and

4) in the immuno-deficient nude mouse, grafted NT2N cells survive >12 months, continue to express N-CAMs and NF proteins, and progressively mature such that they acquire a fully mature neuronal phenotype by 12 months post-transplant.

Of the 5 rats that received subcutaneous cyclosporine, all 5 contained viable NT2N grafts at post-transplant intervals that ranged from 2 to 12 weeks. In contrast, administration of cyclosporine by gavage at the same dose (i.e. 7-10 mg/kg) appeared less effective in preventing graft rejection since only 2 of 8 rats treated in this manner contained an identifiable NT2N graft (Table 1). Notably, the administration of cyclosporine to these rats did not appear to have any detectable effect on the ability of the surviving NT2N cells to express a range of neuronal polypeptides.

Maturation Of Grafted NT2N Cells And The Establishment Of Neuronal Polarity:

Presumably, as a result of their progressive maturation *in vivo*, NT2N grafts that survived 2-4 weeks post-transplantation were the largest and the most amenable to serial section immunohistochemical analysis, while only a limited number of sections containing identifiable NT2N cells could be obtained from rats that survived 4 days to 1 week post-transplantation. For this reason, studies were focused on the maturational state and polarity of the NT2N cells on rats that survived 2-4 weeks post-transplantation. At these time points, NT2N cells in hippocampus or in the subcortical white matter (which consistently contained larger populations of NT2N cells than the neocortex perhaps due to leakage of the NT2N cells from the cortical injection site into the overlying subarachnoid space) expressed several well characterized polypeptides (e.g. NF subunits and other neuronal

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cytoskeletal proteins, synaptic polypeptides) that unequivocally identified the NT2N cells as neurons (see Figures 3A-3E, Figure 3G, Figure 3H and Table 2). However, these neurons resembled late fetal human spinal cord (i.e. >25 weeks gestational age) or young postnatal human cerebellar (i.e. <1 year old) neurons rather than fully mature neurons of the adult CNS in that they failed to acquire heavily phosphorylated isoforms of NF-H. In contrast, polypeptides expressed by glial cells were infrequent in these grafts and the presence of rare GFAP positive astrocytes in these grafts (Figure 1H) undoubtedly reflects the migration of reactive rat astrocytes into the grafts.

Four week old NT2N neurons extended axons over several millimeters (Figures 1B-1F), and some of these axons projected to the hemisphere contralateral to the graft site (Figures 3A-3D). Although dendrites were readily identified because they could be labeled with antibodies to microtubule associated proteins (MAPs) restricted to the somatodendritic domain (e.g. MAP2), these dendrites were short with a simplified branching pattern (Figure 3E). Nonetheless, the presence of identifiable axons and dendrites containing polypeptides that were compartmentalized like their counterparts in authentic rat or human neurons *in vivo* (Figures 1B-1F and Figures 3A-3E, Figure 3G and Figure 3H) indicate that by 4 weeks post-transplantation the grafted NT2N neurons had acquired the molecular phenotype and structural polarity seen in nearly mature human CNS neurons *in vivo*. Further, none of the grafted NT2N cells expressed proteins (e.g. nestin, vimentin, p75 NGFR) that are found in neuronal progenitor cells or very immature (i.e. "nascent") human CNS neurons. Significantly, despite evidence of neuronal degeneration due to graft rejection (Figures 2A-2D), none of the grafts showed evidence of neuronal cytoskeletal protein abnormalities similar to those seen in common neurodegenerative diseases. Finally, there was no evidence (e.g. mitoses, metastases) to indicate that any of the surviving NT2N cells reverted to a neoplastic phenotype.

DISCUSSION

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This study demonstrates the properties of CNS transplants of pure populations of clonal human neuron-like cells that are capable of undergoing progressive, normal maturation and integration into the host mammalian brain without evidence of tumor formation. Only one other CNS cell line, the human HCN-1 line, appears to exhibit an exclusive *in vitro* commitment to the neuronal lineage, but this cell line does not maintain a stable neuronal phenotype when transplanted into the CNS of experimental animals (Ronnett, C.V. et al. 1990 *Science* 248:603-605.

The paucity of suitable neuronal cell lines for transplantation has limited studies of the immunological response of the CNS to transplanted neurons alone. This report demonstrates that the NT2N neurons are capable of expressing antigens that induce rejection by about 4 weeks post-transplant. Although the precise nature of these antigens in the NT2N cells is unknown at this time, human teratocarcinoma cell lines similar to the NT2 parent cell line have been shown to express major histocompatibility antigens such as HLA-A, B and C antigens and  $\beta_2$  microglobulin.

More significantly, this study demonstrates that transplanted NT2N neurons are capable of undergoing partial neuronal maturation in the rat brain. The NT2N cells injected into the rat brain progressively matured to about the same extent as their *in vitro* counterparts maintained in culture for up to 28 days following Replate 3. However, they did not attain the same level of maturity as transplanted NT2N cells that survived for >9-12 months in the immunodeficient nude mouse brain. Specifically, the NT2N grafts in the rat brain did progress to a level of maturation by 4 weeks post-transplant that corresponded to the maturational state of authentic human neurons in the late embryonic spinal cord or in the immature, young postnatal cerebellum. They differ significantly from olfactory sensory neurons and the neuron-like tumor cells in CNS medulloblastomas. However, the transplanted and cultured NT2N cells do resemble the

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differentiated and fairly mature neurons that have been observed *in situ* in some teratocarcinomas and many teratomas.

Based on the findings presented here, the transplantation of the NT2N cells into experimental animals can be exploited for several types of unique studies of the developmental biology of neurons and the regressive neurodegenerative events that occur in some neurological disorders. First, the ability to "re-start" the process of neuronal maturation and the development of neuronal polarity by transplanting the NT2N cells into different regions of the rodent brain can be used as models of these two important developmentally regulated processes. The availability of an effective human model system to study these processes in a controlled experimental setting should greatly facilitate efforts to gain insights into the regulatory mechanisms that govern these processes. This model system also will allow the opportunity to explore the possibility that the micro-environment of the host brain might induce NT2N cells grafted into different neuroanatomical sites to assume a region specific morphological and neurotransmitter phenotype.

Moreover, wild type or genetically modified NT2N cells can be used to develop animal models of humans diseases, conditions and disorders, particularly nervous system diseases. For example, the NT2N cells preferentially express the 695 amino acid long Amyloid precursor protein (APP<sub>695</sub>) and they secrete the  $\beta$ /A4 peptide into the culture medium. Hence, the wild type NT2N cells, NT2N cells transfected to overexpress APP<sub>695</sub>, or NT2N cells transfected to overexpress  $\beta$ /A4 can be transplanted in order to provide an animal model that releases APP<sub>695</sub> or  $\beta$ /A4 into the extracellular space following transplantation. The deposition of  $\beta$ A4 peptides that occur in the Alzheimer's disease brain can be modelled in this way.

Transplantation of NT2N cells genetically engineered to produce bioactive molecules can be used to develop novel methods to circumvent the blood-brain barrier for the treatment of human neurodegenerative diseases. For example, in view of the therapeutic promise evidenced by recent studies of the use

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of fetal mesencephalic grafts for the treatment of Parkinson's disease, induction of NT2N cells to acquire a dopaminergic phenotype for use in the treatment of Parkinson's disease followed by transplantation can be a therapy for individuals  
5 suspected of suffering from Parkinson's disease.

**Example 2****Transfection and Staining for  $\beta$ -galactosidase:**

Highly purified populations of neurons from a human teratocarcinoma cell line were obtained as described in U.S.  
10 Patent Number 5,175,103. When undifferentiated, the NT2 cells were transfected with 100  $\mu$ g SPUD1 and 10  $\mu$ g of pSV2neo by lipofection using LIPOFECTIN transfecting reagent (Bethesda Research Laboratories). SPUD1 is a  $\beta$ -galactosidase expression vector which utilizes the SV40 promoter and has Moloney murine  
15 leukemia virus long terminal repeats upstream and downstream. After two days in complete medium, the transfectants were selected with 200  $\mu$ g/ml G418 (Gibco) for seven days. Cells were stained for  $\beta$ -galactosidase activity with 1 mg/ml X-gal, 5mM potassium ferrocyanide, 5mM potassium ferricyanide, 2mM  
20  $MgCl_2$  in PBS after fixation in 2% paraformaldehyde, 0.2% glutaraldehyde in phosphate buffered saline pH 7.4.  $\beta$ -gal positive cultures were subcloned twice and the subclones were used for further studies. The cells were photographed using Hoffman modulation contrast to allow the simultaneous  
25 visualization of the blue reaction product and the processes.

The  $\beta$ -galactosidase ( $\beta$ -gal) expression plasmid was shown to be present in both undifferentiated and post-mitotic cells. Thus, transfection of expression plasmids into undifferentiated cells allows the introduction of exogenous  
30 genetic material into cells. The cells can then be induced to become stable, post-mitotic human neurons and can express the exogenous genetic material.

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TABLE 1

## POST-TRANSPLANTION SURVIVAL DATA FOR GRAFTED NT2N CELLS

	Post-transplant survival	Number Of Rats injected w/NT2N Cells	Number Of Rats with viable NT2N cell grafts
5			
		<u>ADULT UNTREATED RATS</u>	
	4 Days	3	3
	2 Weeks	2	2
	4 Weeks	24	10
10	6 Weeks	4	0
	8 Weeks	3	1
	13 Weeks	2	0
	SUBTOTAL	= 38	SUBTOTAL = 16
		<u>NEONATAL UNTREATED RATS</u>	
15	1 Week	2	2
	2 Weeks	3	3
	4 Weeks	2	2
	8 Weeks	2	0
	12 Weeks	2	0
20	16 Weeks	2	0
	16 Weeks	2	0
	21 Weeks	2	0
	SUBTOTAL N	= 17	SUBTOTAL N = 7
		<u>ADULT CYCLOSPORINE TREATED RATS</u>	
25	2 Weeks	3 (sc = 1)	3 (sc = 1)
	4 Weeks	1 (sc)	1
	6 Weeks	1 (sc)	1
	8 Weeks	2 (sc = 1)	1 (sc = 1)
	10 Weeks	2	0
30	11 Weeks	3	0
	12 Weeks	1 (sc)	1
	SUBTOTAL	= 13	SUBTOTAL = 7
	GRAND TOTAL	= 68	GRAND TOTAL = 30

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TABLE 2

POLYPEPTIDE AND CELL SPECIFICITY OF ANTIBODIES AND THEIR  
REACTIVITY WITH GRAFTED NT2N CELLS

5	POLYPEPTIDE	ANTIBODY	DILUTION $\mu\text{g/ml}$	NT2N GRAFT
NEURONS				
	Clathrin light chain	LCB2	0.1	+/-
	MAP2	AP14	1:100	+
10	MAP5	AA6	1:1500	+
	NF-H, P <sup>+++</sup>	RMO24	Neat	-
	NF-H, P <sup>+++</sup>	RMO217	Neat	-
	NF-H, P <sup>+</sup>	TA51	1:20	+
	NF-H/M, P <sup>-</sup>	RMdO20	1:10	+
15	NF-H, P <sup>++</sup>	HO14	1:25	+
	NF-M, p <sup>ind</sup>	RMO254	1:25	+
	NF-L, p <sup>ind</sup>	NR 4	1:10	+
	NF-L, p <sup>ind</sup>	Anti-NF-L	1:50	+
	Neuron Specific Protein	NST11	1:10	-
20	Protein Kinase C $\gamma$	PKC66	Neat	-
	Tau	T14	Neat	+
	Tau	134	1:500	+/-
	Tau (fetal/PHF)	T3P	1:50	+
	Tau (fetal/PHF)	PHF1	1:2000	+/-
25	NEURONS AND NEUROENDOCRINE CELLS			
	Chromogranin	LK2h10	1:500	-
	Synaptophysin	SY 38	1:100	+/-
	Tyrosine Hydroxylase	Anti-TH	1:100	-
30	NEUROEPITHELIAL STEM CELLS			
	Nestin	Anti-Nestin	1:2000	-
GLIAL CELLS				
	GFAP	2.2B10	1:500	-
	MBP	Anti-MBP	1:100	-
35	NEURAL, MESENCHYMAL & OTHER CELLS			
	N-CAM	MOC 1	1:10	+
	NGFR	Me 20.4	1:100	-
	Vimentin	V9	1:100	-
	Macrophage marker	ED1	1:500	-

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## CLAIMS

1. A method of treating an individual suspected of suffering from a central nervous system disease or disorder  
5 comprising the step of:

implanting a sample from a culture of at least 95% pure, stable, homogeneous post-mitotic human neurons into said individual's brain.

2. The method of claim 1 wherein said neurons are  
10 differentiated NT2N cells.

3. The method of claim 1 wherein said neurons are transfected, differentiated NT2N cells that have been transfected with exogenous genetic material, wherein said transfected, differentiated cells express coding sequences of  
15 said exogenous genetic material to produce a protein product.

4. The method of claim 3 wherein said exogenous genetic material includes nucleic acid sequences that encode proteins selected from the group consisting of neurotransmitters and neurotrophic substances such as nerve  
20 growth factor, brain-derived neurotrophic factor (BDGF), basic fibroblast growth factor (bFGF) and glial-derived growth factor.

5. The method of claim 1 wherein said central nervous system disease or disorder is selected from the group  
25 consisting of Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and nerve injuries.

6. A pharmaceutical composition comprising:  
a sample from a culture of at least 95% pure, stable, homogeneous post-mitotic human neurons; and  
30 a pharmaceutically acceptable medium.

7. The pharmaceutical composition of claim 6 wherein said neurons are differentiated NT2N cells.

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8. The pharmaceutical composition of claim 6 wherein said neurons are transfected, differentiated NT2N cells that have been transfected with exogenous genetic material; wherein transfected, differentiated cells express coding sequences of  
5 said exogenous genetic material to produce a protein product.

9. The pharmaceutical composition of claim 6 wherein said exogenous genetic material includes nucleic acid sequences that encode a protein selected from the group consisting of neurotransmitters (e.g. tyrosine hydroxylase) and neurotrophic  
10 substances such as nerve growth factor, brain-derived neurotrophic factor (BDGF), basic fibroblast growth factor (bFGF) and glial-derived growth factor.

10. A method of generating a non-human animal model for a central nervous system disease or disorder comprising the  
15 step of:

implanting a sample from a culture of at least 95% pure, stable, homogeneous post-mitotic human neurons into said non-human animal's brain.

11. The method of claim 10 wherein said neurons are  
20 NT2N cells.

12. The method of claim 10 wherein said neurons are NT2N cells that have been transfected with exogenous genetic material, wherein transfected cells express coding sequences of said exogenous genetic material to produce a protein product.

25 13. The method of claim 10 wherein said exogenous genetic material includes nucleic acid sequences that encode proteins selected from the group consisting of normal and mutated amyloid precursor, kinases, phosphatases, normal and mutated superoxidedismutase, neurofilament proteins and  
30 apolipoprotein 4.

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14. The method of claim 10 wherein said disease or disorder is selected from the group consisting of Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and nerve injuries.

5           15. The method of claim 10 wherein said animal is a rodent.

16. The method of claim 10 wherein said animal is an immunocompetent rodent and further comprising the step of administering Cyclosporin to said rodent before, during and/or  
10 after implanting said cells.

17. An non-human animal comprising:  
a sample from a culture of at least 95% pure, stable, homogeneous post-mitotic human neurons implanted in the brain of said non-human animal.

15           18. The non-human animal of claim 17 wherein said neurons are NT2N cells.

19. The non-human animal of claim 17 wherein said neurons are NT2N cells that have been transfected with exogenous genetic material, wherein transfected cells express  
20 coding sequences of said exogenous genetic material to produce a protein product.

20. The non-human animal of claim 19 wherein said exogenous genetic material includes nucleic acid sequences that encode proteins selected from the group consisting of normal  
25 and mutated amyloid precursor, kinases, phosphatases, normal and mutated superoxidedismutase, neurofilament proteins and apolipoprotein 4.

21. A method of treating an individual suspected of suffering from an injury, disease or disorder characterized by  
30 nerve damage comprising the step of:

- 34 -

implanting a sample from a culture of at least 95% pure, stable, homogeneous post-mitotic human neurons at or near a site of said nerve damage.

22. The method of claim 21 wherein said neurons are  
5 differentiated NT2N cells.

23. The method of claim 21 wherein said injury is a spinal injury and said sample is implanted in said individual's spinal column.

24. The method of claim 1 wherein said injury is to  
10 a motor neuron.

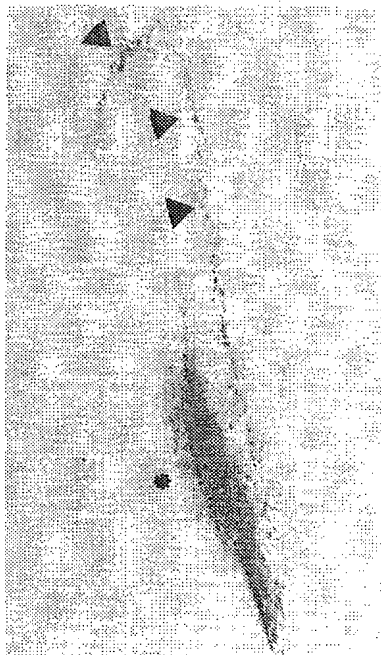


FIG. 1B



FIG. 1D

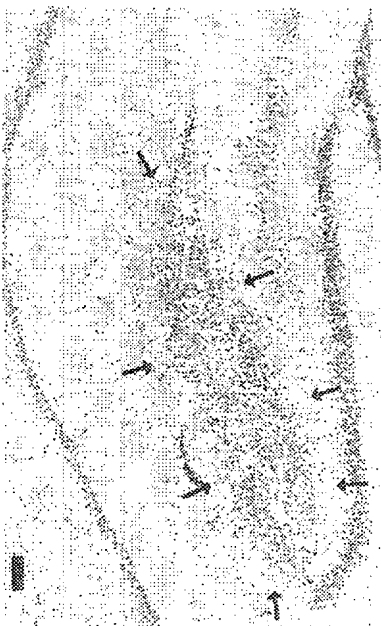


FIG. 1A

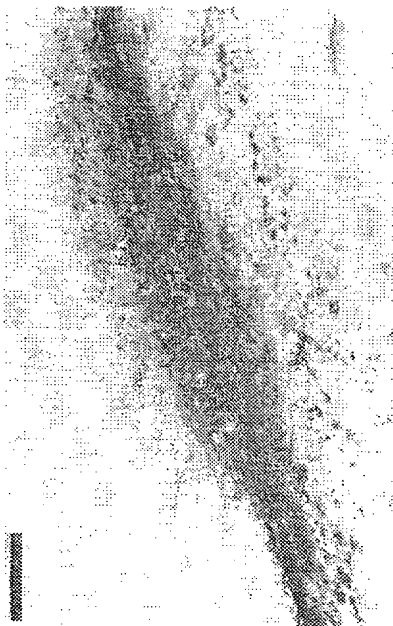


FIG. 1C

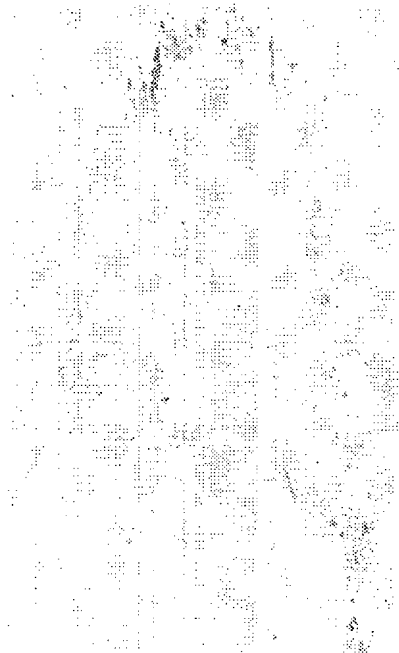


FIG. 1F

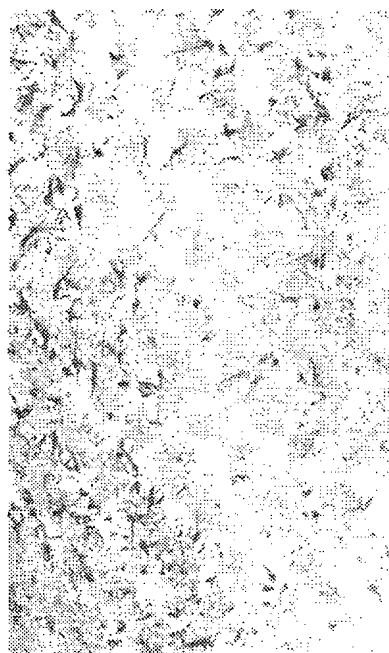


FIG. 1H



FIG. 1E

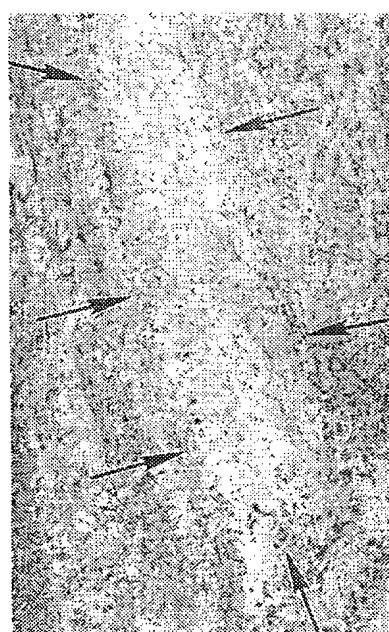


FIG. 1G

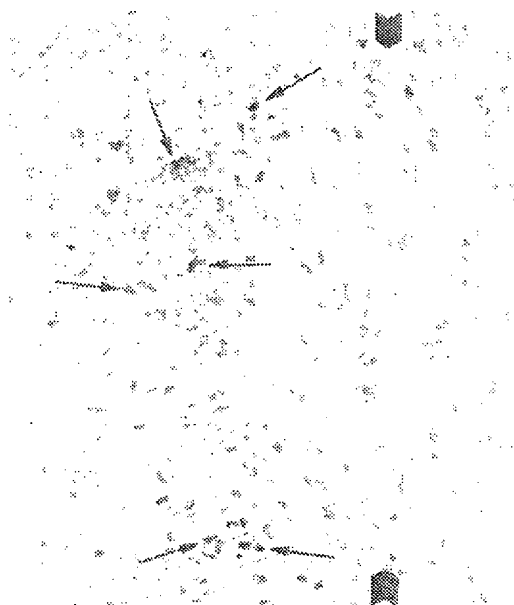


FIG. 2B



FIG. 2D

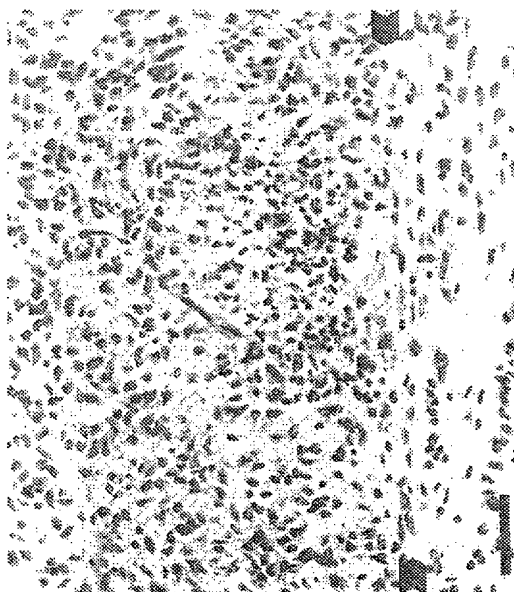


FIG. 2A

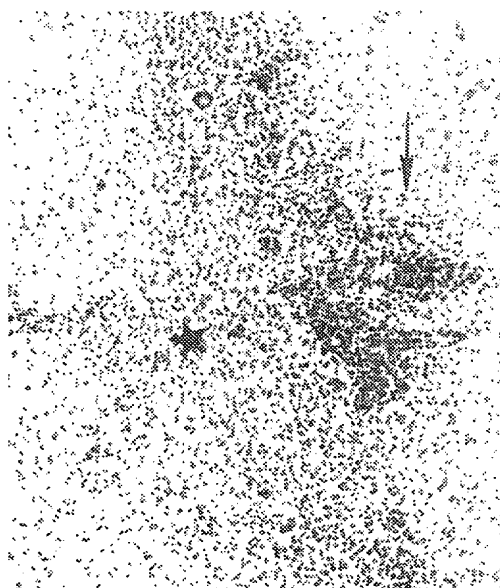


FIG. 2C

4/5

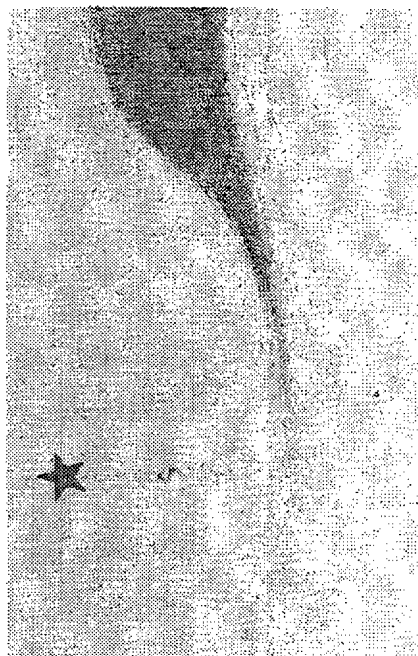


FIG. 3B



FIG. 3D

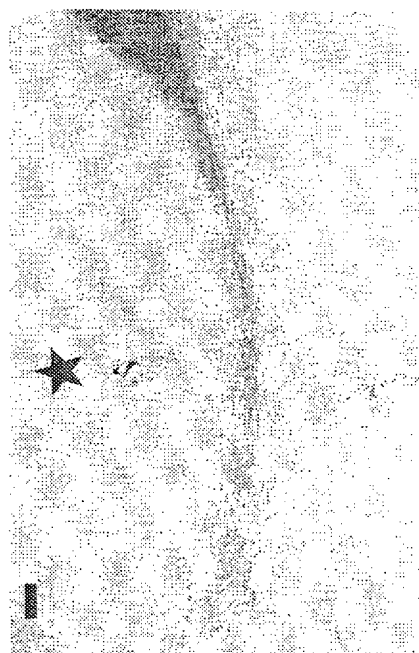


FIG. 3A

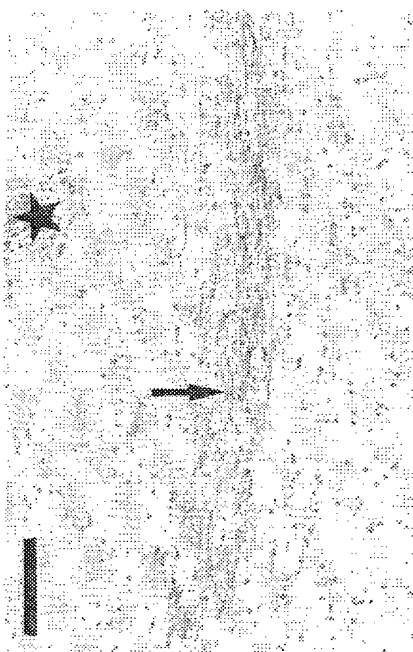


FIG. 3C



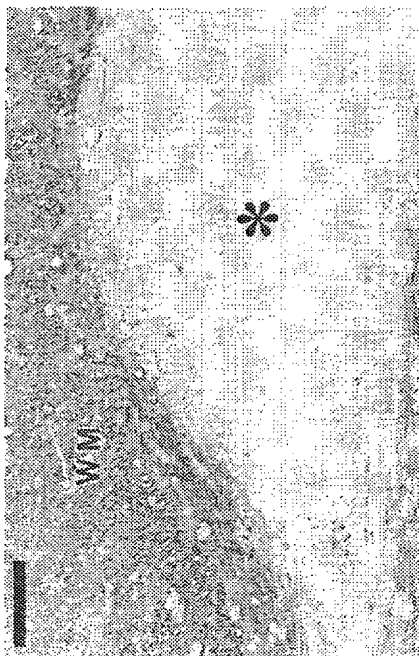


FIG. 3F



FIG. 3H

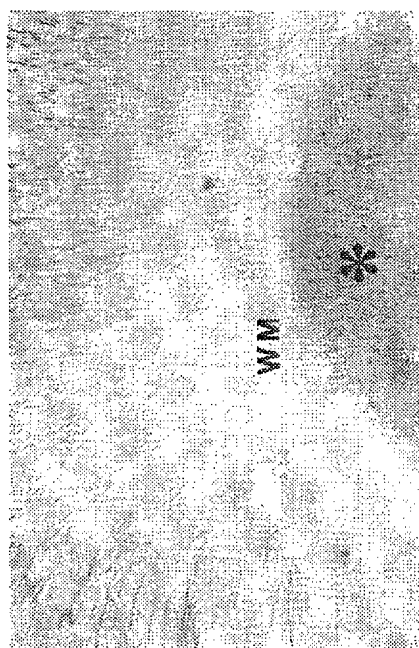


FIG. 3E

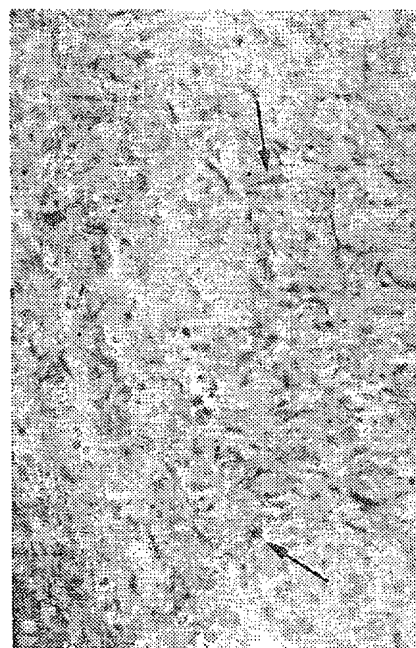


FIG. 3G

## INTERNATIONAL SEARCH REPORT

 International application No.  
 PCT/US94/12899
**A. CLASSIFICATION OF SUBJECT MATTER**
 IPC(6) : A01N 63/00; A61K 48/00; C12N 5/00, 5/06, 5/08, 15/00  
 US CL : 424/9, 93.1, 93.2, 93.7; 435/172.1, 172.3, 240.2, 240.1,  
 According to International Patent Classification (IPC) or to both national classification and IPC
**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/9, 93.1, 93.2, 93.7; 435/172.1, 172.3, 240.2, 240.1,

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,175,103 (LEE ET AL.) 29 DECEMBER 1992, see entire document.	1-9 and 21-24
Y	US, A, 5,180,820 (BARDE ET AL.) 19 JANUARY 1993, see entire document.	1-9 and 21-24
Y	US, A, 4,892,538 (AEBISCHER ET AL.) 09 JANUARY 1990, see entire document.	1-9, 21-24
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, Volume 86, issued June 1989, P. Ernfors et al., "A cell line producing recombinant nerve growth factor evokes growth responses in intrinsic and grafted central cholinergic neurons", pages 4756-4760, see entire document.	1-9 and 21-24



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

23 JANUARY 1995

Date of mailing of the international search report

13 FEB 1995

 Name and mailing address of the ISA/US  
 Commissioner of Patents and Trademarks  
 Box PCT  
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

BRIAN R. STANTON

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/12899

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NEW ENGLAND JOURNAL OF MEDICINE, Volume 327, Number 22, issued 26 November 1992, C. R. Freed et al., "Survival of implanted fetal dopamine cells and neurologic improvement 12 to 46 months after transplantation for parkinson's disease", pages 1549-1555, see entire document.	1-9 and 21-24.
Y	THE NEW ENGLAND JOURNAL OF MEDICINE, Volume 327, Number 22, issued 26 November 1992, D.D. Spencer et al., "Unilateral transplantation of human fetal mesencephalic tissue into the caudate nucleus of patients with parkinson's disease", pages 1541-1548, see entire document.	1-9 and 21-24
X ----- Y	EXPERIMENTAL NEUROLOGY, Volume 122, issued August 1993, J.W. Trojanowski et al., "Neurons derived from a human teratocarcinoma cell line establish molecular and structural polarity following transplantation into the rodent brain", pages 283-294, see entire document.	1-3, 6, 7 ----- 5, 8, 9 and 21-24
Y	NATURE, Volume 362, issued 01 April 1993, S. Jiao et al., "Long-term correction of rat model of Parkinson's disease by gene therapy", pages 450-453, see entire document.	1-9 and 21-24

**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

Databases: APS, AQUASCI, BIOBUSINESS, BIOSIS, BIOTECHDS, CA, CABA, CANCERLIT, CAPREVIEWS, CEN, CIN, CJACS, CJELSEVIER, CONFSCI, DISSABS, DRUGB, DRUGLAUNCH, DRUGNL, DRUGU, EMBASE, FSTA, GENBANK, HEALSAFE, IFIPAT, JICST-E, JPNEWS, LIFESCI, MEDLINE, NTIS, OCEAN, PHIC, PHIN, PROMT, SCISEARCH, TOXLINE, TOXLIT, USPATFULL

Search Terms: Lee?/au; neuro?; nt2n; implant?; transfect?; brain; derived; neurotrophic; bdnf; bdgf; basic; fibroblast?; glial?; alzheimer?; parkinson?; huntington?; transplant?; cell?

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-9 and 21-24, drawn to methods of treating neurological disorders.

Group II, claims 10-16, drawn to methods of preparing animal model systems.

Group III, claims 17-20, drawn to non-human animals.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The inventions of groups I and II are distinct, one from the other, because they represent materially different methods of using cellular compositions. In the case of the invention of group I, the cells are used to treat neurologic disorders. Therefore, the cellular compositions are required to ameliorate disease conditions. In the case of the invention of group II, the cells administered to the animal are used to induce a disease condition in the host. Therefore, two disparate and diametrically opposite uses of cellular compositions are indicated by the two claimed inventions.

The animals of the invention of group III are distinct from the invention of group I because said animals represent a distinct composition from that claimed by the latter invention. In the case of group I, cellular compositions are claimed whereas in the case of group III, modified multicellular animals are claimed.

The methods of the invention of group II are distinct from the animals of the invention of group III because the former methods are drawn to means of producing animal models of disease and therefore require search and consideration of disease systems. In contrast, the animals of the invention of group III are not required to comprise any model systems and therefore consideration of disease states, per se, is not required.

For the preceding reasons, the above listed inventions are not so linked by any special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.